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RESEARCH PAPERS

Virus diseases of vegetable crops in southern Bulgaria

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Summary. Virus diseases of vegetable crops (mainly tomato, cucumber, pepper and phaseolus bean) were surveyed in 1999 in south-eastern Bulgaria. The most widespread viruses were *Tomato mosaic virus* (ToMV), *Cucumber mosaic virus* (CMV) and *Pepper mild mottle virus 1.2* (PMMV 1.2) for pepper; ToMV, CMV and *Tomato spotted wilt virus* for tomato; CMV for cucumber; *Bean common mosaic virus* (BCMV) for bean. Using differential cultivars as well as ELISA and PCR, the viruses were classified into pathotypes, strains and subgroups. The CMV isolates were of subgroup I, Iγ and II. The BCMV isolates were identified as temperature-dependent necrotic strains. *Bean common mosaic necrosis virus* was found in only two samples. PMMV 1.2 was detected in pepper. *Clover yellow vein virus* in bean and *Cucumber leaf spot virus* in cucumber are here reported for the first time in Bulgaria. A cucumovirus, different from CMV, was detected in bean. Two unidentified viruses (one with capillovirus-like particles from pepper, and one with 30 nm spherical particles from cucumber) are currently under investigation.

Key words: viruses, phaseolus bean, cucumber, pepper, tomato.

Introduction

Vegetable crops are important in Bulgaria. In recent years about 12,570 ha have been occupied by vegetables, mainly tomato (22%), pepper (17%), onion (11%), cucumber (9%) and phaseolus bean (4.7%). These crops are generally grown in the open, with a comparatively small acreage in tunnels or greenhouses. Most vegetable crops are located in south-eastern Bulgaria, the so-called "Bulgarian vegetable garden", where mild climate and water availability favour production. Unfortunately this production is often limited by pests and diseases, among which viruses seem to play an important role. Earlier surveys on viruses of vegetables in Bulgaria were conducted by Kovachevski (1965, 1971), Kovachevski *et al.* (1977) and Kostova and Dimitrov (1995).

The investigation here reported was carried out in 1999, mainly on viruses in four crops (cucumber, pepper, phaseolus bean and tomato) grown in south-eastern Bulgaria. The new data will be used in the national breeding programme aimed at creating virus-resistant vegetable lines and cultivars. Some of the results were briefly reported earlier (Tsorlianis and Kostova, 2000; Tsorlianis *et al.*, 2000; Kostova *et al.*, 2001).

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Materials and methods

Collection of virus samples

A total of 102 samples from different crops were collected; of these 18 were from cucumber, 25 from phaseolus bean, 38 from pepper, 17 from tomato, three from melon and one from eggplant. For each crop, except melon and eggplant, samples were collected in at least four locations. Samples were collected in June 1999 and consisted of leaves taken from plants with symptoms suggesting virus infection. Leaves were placed in plastic bags and stored in a cool bag in the field. Upon arrival in the laboratory at the Institute for Horticultural and Canned Food in Plovdiv, samples were divided in two parts: one was desiccated over $CaCl_2$, and the other under vacuum. All desiccated samples were then taken to the Istituto di Virologia Vegetale (IVV) (formerly Istituto di Fitovirologia Applicata) in Torino, Italy, where they were analysed under strict phytosanitary control.

Isolation and host-ranges

To prepare inoculum, the dried samples were ground in ice-cold 50 mM phosphate buffer, pH 7, containing 5 mM Na-DIECA, 5 mM Na-thioglycolate, 1 mM Na-EDTA and about 10 mg ml^{-1} activated charcoal. The sap was rubbed on carborundum-dusted leaves of *Chenopodium quinoa, Nicotiana benthamiana, N. clevelandii* and 'White Burley' tobacco for all samples, plus *Capsicum annuum* cv. Quadrato d'Asti for pepper samples, *Cucumis sativus* cv. Marketer and *Cucurbita pepo* cv. Genovese for cucumber, *Lycopersicum esculentum* cv. Marmande for tomato and *Phaseolus vulgaris* cv. Saxa for bean.

Viruses were isolated from mixed infections by differential reactions of the host plants or, for some viruses, by thermal inactivation (10 min at 75°C) of the more unstable virus.

Capsicum spp. differentials (*C. annuum* 'Bruinsma Wonder', *C. frutescens* 'Tabasco', *C. chinense* PI 159236 and *C. chacoense* PI 260409) were used to pathotype tobamoviruses (Rast, 1988), and bean differentials (*P. vulgaris* 'Widusa', 'Jubila', 'Amanda' and IVT 7233) to differentiate *Bean common mosaic virus* (BCMV) pathotypes (Morales and Bos, 1988) and to confirm the identification of *Bean common mosaic necrosis virus* (BCMNV).

Serological tests

Original desiccated samples of each crop were tested for several viruses by different ELISA procedures, as indicated in Table 1. ELISAs were performed according to standard procedures (Converse and Martin, 1990), with dilution of reagents according to manufacturer's instructions or IVV protocols. Reactions were considered positive when the infected/healthy ratio was 3 or more.

Agar gel diffusion (done according to Purcifull and Batchelor, 1977, for the elongated viruses) was used for the detection of some viruses in sap from experimentally infected plants. Antisera to BCMNV, *Broadbean wilt virus*-1 and -2 (BBWV-1, -2), *Clover yellow vein virus* (ClYVV) and *Tomato bushy stunt virus,* were from the IVV collection; antisera to *Cucumber leaf spot virus* (CLSV) was from D. Gallitelli, Bari, Italy, to *Cucumber necrosis virus* from DSMZ, Braunschweig, Germany, to *Cucumber soil-borne virus* from R. Koenig, Braunschweig, Germany, and to *Sowbane mosaic virus* (SoMV) from C.I. Kado, Riverside, CA, USA.

Electron microscopy

Extracts of original desiccated samples and experimentally infected indicator plants were tested by negative staining with uranyl acetate.

RT-PCR analysis of CMV isolates

Approximately 0.2 g leaf tissue from experimentally infected test plants of selected samples were processed with the RNAWIZ reagent (Ambion, Austin, TX, USA) according to manufacturer's instructions, to obtain total RNA. The RNA pellets were resuspended in 40 μl diethyl pyrocarbonate-treated water and 1 μl was used as template for reverse transcription. Complementary DNA synthesis and PCR were performed as described in Anonymous (1998), using 5'CP and 3'CP primers (Rizos *et al.,* 1992) and *Msp*I digestion of the amplicons. After enzymatic digestion, the DNA was electrophoresed in 3% NuSieve 3:1 agarose (FMC, Rockland, ME, USA) and stained with ethidium bromide.

Results

A total of 102 samples were tested. The viruses detected, both in the original desiccated material by ELISA and those recovered from experimental-

Table 1. Antisera or antibodies used to detect viruses by ELISA in the original desiccated samples.

Crop	Virus	Type of ELISA and reagents
Cucumber and melon	*Cucumber mosaic (*CMV*)*	TAS, coating: sera to subgroup I + II[a]; Mabs: 2+85[b]; Mabs subgroup I/II[c]
	Cucurbit aphid-borne yellowing	DAS, kit supplied by H. Lecoq, Montfavet, France
	Moroccan watermelon mosaic	DAS, serum supplied by Dr S. Winter, Brauschweig, Germany, purified and conjugated at IVV
	Papaya ringspot	DAS[a]
	Tobacco necrosis (TNV)	DAS[d]
	Squash mosaic	DAS, Loewe Biochemica GmbH, Sauerlach, Germany
	Watermelon mosaic (WMV)	DAS[a]
	Zucchini yellow fleck	DAS[a]
	Zucchini yellow mosaic	DAS[a]
	Watermelon silver mottle	TAS, coating[a], Mab 1B4[e]
Bean	*Alfalfa mosaic* (AMV)	DAS, Loewe Biochemica GmbH
	Bean common mosaic (BCMV)	DAS[a]
	Bean yellow mosaic	DAS, Loewe Biochemica GmbH
	CMV	TAS, coating: sera to subgroup I + II[a]; Mabs: 2+85[b]; Mabs subgroup I/II[c]
	TNV	DAS[d]
	Cucumovirus Ch39	DAS[a]
Pepper	AMV	DAS, Loewe Biochemica GmbH
	CMV	TAS, coating: sera to subgroup I + II[a]; Mabs: 2+85[b]; Mabs subgroup I/II[c]
	Pepper mild mottle (PMMV) 1,2,3	DAS[a f]
	Potato virus Y (PVY)	DAS[a]
	Tomato mosaic (ToMV)	DAS[a g]
	TNV	DAS[d]
	Tomato spotted wilt (TSWV)	TAS, coating: sera [a]; Mab NUV2[e]
Tomato	As for pepper, plus	
	Tomato infectious chlorosis	DAS[a h]
	Parietaria mottle-T	ACP[a i]

[a] Collection of the Istituto di Virologia Vegetale, CNR, Torino, Italy.
[b] Grassi *et al*., 1995.
[c] AGDIA kit able to differentiate CMV subgroups I and II.
[d] Roggero and Lisa, 1995.
[e] Roggero *et al*., 1998.
[f] Serum able to detect all pepper tobamoviruses except P 1.
[g] Serum able to detect pepper tobamovirus P 0.
[h] Dellavalle *et al*., 1995.
[i] Lisa *et al*., 1998.

ly infected indicator plants, are listed in Table 2. Most data obtained on the original material by ELISA could be confirmed by infectivity tests, with the possible exception of *Alfalfa mosaic virus* (AMV), for which no good correlation between ELISA and infectivity was found. No appreciable difference was found between samples desiccated under vacuum and those treated with $CaCl_2$. A number of samples were negative by ELISA for the viruses tested, but infectivity tests showed them to be infected with other viruses such as BBWV-1, ClYVV, or two unidentified viruses. Some viruses, especially potyviruses such as *Potato virus Y* (PVY), *Watermelon mosaic virus* (WMV) in one case, or an unidentified potyvirus detected in eggplant by ELISA, could not be isolated on the indicators. As expected, *Tomato spotted wilt virus* (TSWV) infectivity did not survive in the desiccated leaves. About

19% of the samples were infected by two or more viruses, while 23% of samples were negative both in ELISA and in the infectivity tests.

Of the 22 CMV isolates detected by ELISA and the infectivity tests (Table 2), seven were further characterised by RT-PCR. Of these, five were identified as subgroup I and two as subgroup Iγ (Anonymous, 1998). In four bean samples, a cucumovirus similar to the undescribed "cucumovirus Ch39", found in 1990 in bean in northern China, was detected by ELISA. This virus was related to, but different from CMV and from a strain of *Peanut stunt virus* and *Tomato aspermy virus* (P. Roggero, G. Dellavalle, V. Lisa and F. Morales, unpublished data). In two of these isolates tested by RT-PCR, the 870 bp amplicon expected for CMV samples was not obtained.

Tobamoviruses infecting pepper and tomato were identified as *Pepper mild mottle virus 1.2* (PMMV 1.2) or *Tomato mosaic virus* (ToMV). BCMV was the commonest potyvirus detected in bean.

Heat inactivation of CMV or WMV in doubly-infected indicators permitted the isolation of CLSV and of an unidentified virus from cucumber with spherical 30 nm particles, currently under study at IVV.

A virus with a flexuous capillovirus-like structure, not yet identified, was found in pepper. In experimental conditions this virus caused lethal necrosis on every *Capsicum* species or *C. annuum* cultivar tested. This virus is also currently under study at IVV.

Discussion

The viruses detected on vegetables in Bulgaria in this investigation are generally common and widespread in temperate regions. Some of them, such as BCMV in bean, or ToMV in pepper and tomato, were also found to be widespread in the same crops in Bulgaria by Kovachevski (1965) and Kowachevski *et al.* (1977), indicating that some of the virus problems have not changed substantially over the last 30 years.

Considering the different crops, the commonest virus in phaseolus beans in our samples was

Table 2. Viruses detected in 1999 in the desiccated field samples by ELISA and recovered on experimentally infected plants. Several samples were infected with two viruses.

Crop	Desiccated samples (detection by ELISA)		Isolation on test plants	
	No.[a]	Virus[b]	No.[a]	Virus[b]
Cucumber	18	CMV[c] (13), WMV (3), negative (4)	18	CMV (12), CLSV (3), WMV (1), SoMV (1), unidentified 30 nm virus (2), negative (3)
Bean	25	BCMV (11), CMV (7), cucumovirus Ch39 (4), negative (8)	25	AMV (2), BCMV (13), BCMNV (2), CMV (6), ClYVV (1), cucumovirus Ch39 (4), negative (3)
Pepper	38	AMV (7), CMV (5), tobamoviruses (15), TSWV (4), negative (9)	38	AMV (1), BBWV1 (3), CMV (3), PMMV 1.2 (4), ToMV (15), unidentified capillo-like virus (1), negative (13)
Tomato	17	CMV (1), PVY (2), ToMV (3), TSWV (7)	13	CMV (1), ToMV (4), unidentified carla-like virus (2), negative (6)
Melon	3	CMV (1)	2	Negative (2)
Eggplant	1	AMV, unidentified potyvirus	1	AMV

[a] No. of samples tested.
[b] In brackets No. of samples found infected or negative.
[c] CMV, *Cucumber mosaic virus*; WMV, *Watermelon mosaic virus*; CLSV, *Cucumber leaf spot virus*; SoMV, *Sowbane mosaic virus*; BCMV, *Bean common mosaic virus*; AMV, *Alfalfa mosaic virus*; BCMNV, *Bean common mosaic necrosis virus*; ClYVV, *Clover yellow vein virus*; BBWV1, *Broad bean wilt virus 1*; TSWV, *Tomato spotted wilt virus*; PMMV 1.2, *Pepper mild mottle virus 1.2*; ToMV, *Tomato mosaic virus*; PVY, Potato virus Y.

BCMV, followed by CMV, BCMNV and AMV. Such a virus-distribution for bean has already been reported in Bulgaria (Kostova *et al*., 1995). BCMV and BCMNV isolates detected in this study were further studied at IHCF (Tsorlianis and Kostova, 2000). The BCMV isolates were identified as temperature-dependent necrotic strains, but were considered to be unique on the basis of their phenotypic expression on bean differential varieties. The BCMNV isolates were similar to the former VIa pathotype of BCMV (Morales and Bos, 1988). Based on PCR results, CMV bean isolates were assigned to subgroup I and subgroup Iγ. The first isolate of CMV from bean to be assigned to subgroup Iγ was also from Bulgaria (Anonymous, 1998). The finding of more Bulgarian CMV isolates belonging to this subgroup suggests that this virus may be endemic in the area, as already noted by Tsorlianis *et al*. (2000). The finding of a cucumovirus similar to isolate Ch39, found in 1990 in northern China, is also of interest. In field beans this virus was associated with severe disease, and it should be further studied. The detection of ClYVV is a new record for Bulgaria.

CMV appeared to be the most widespread virus in cucumber. All isolates tested by RT-PCR belonged to CMV subgroup I. WMV, found in cucumber and in one zucchini sample (data not shown), was already reported in Bulgaria by Dikova *et al*. (1984). Two viruses new for Bulgaria on cucumber were identified by this study: CLSV (Kostova *et al*., 2001) and SoMV. SoMV can be considered an occasional infection since it occurs commonly in weeds. Four original cucurbit samples showing yellowing were tested for cucurbit criniviruses (*Cucurbit yellow stunting disorder virus* and *Lettuce infectious yellows virus*) by RT-PCR with specific primers and found to be negative (A.M. Vaira, data not shown).

In pepper and tomato samples, tobamoviruses, particularly ToMV, and TSWV, were the viruses most frequently detected. ToMV appeared to be a serious problem particularly for pepper, being identified in about 50% of samples. In both these crops CMV was also detected. ELISA of CMV isolates revealed both subgroups, I and II. In pepper, AMV, BBWV-1 and PMMV-1.2 were also identified, at lower percentages. These viruses have already been reported in Bulgaria (Kovachevski, 1976; Yankulova and Kaitazova, 1979; Kostova *et al.,* 1995).

The relatively high number of samples negative both by ELISA done on original material and by the infectivity tests (23%) can be explained by considering that sampling was done early in the season, after an unusually cold spring that had delayed the transplanting of seedlings to the field. Particularly in pepper and tomato crops, the plants were rather young and the symptoms sometimes uncertain. Furthermore, viruses not sap-transmissible to indicators, or for which no detection kit was available at IVV, may have escaped detection.

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